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Grant Number DAMD17-94-J-4230

TITLE: Homebox Genes in Normal, Preneoplastic, and Neoplastic Mammary Glands

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REPORT DATE: December 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1998	Annual (1 Jan 98 – 31 Dec 98)

4. TITLE AND SUBTITLE
Homebox Genes in Normal, Preneoplastic, and Neoplastic Mammary Glands

5. FUNDING NUMBERS
DAMD17-94-J-4230

6. AUTHOR(S)
Dr. Charles W. Daniel

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Santa Cruz, California 95064

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

The overall aim of this project is to improve our understanding of genetic factors regulating the development, differentiation, function, and neoplastic progression of the breat. In the previous year we discovered the first clearly identified mammary phenotype in homeobox genes, an engineered mutation in mouse Hoxd-10 that causes a deficiency in milk production. In 1998 we report the conclusion of this project. More important, we have carried out extensive functional and expression studies on the Hedgehog (Hh) pathway, not previously shown to be active in the breast. This pathway has been shown in several model systems to control many of the signaling pathways known to regulate mammary development, and thus has the potential to be considered a "master regulator." Using gene-targeted mice we have shown dramatic phenotypes associated with partial loss of receptor function and with loss of one of the transcription factors mediating Hh function. The discovery that Hh signaling is essential to mammary development has far-reaching implications for our understanding of both the normal and neoplastic breast.

14. SUBJECT TERMS
Hox, Precancerous, Homeobox, Growth Factors, Hormone, Maligant progression

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

1998 was an exciting research year in this project and we feel that the data generated reach far into the future, and may in fact provide a new paradigm for understanding the genetic regulation of breast development. As indicated in the 1997 progress report, we have continued our research on homeotic genes with the discovery of a mammary phenotype associated with a Hoxd-10 mutation. This work is now completed and a manuscript is in preparation. Interestingly, a recent publication in PNAS (Chen and Capecchi) reproted a similar but weaker phenotype with a triple mutant of Hox-9 genes.

This year, as indicated in in the revised statement of work, we have taken a much more focused approach and concentrated on identification in the mammary gland of a highly expressed upstream regulatory pathway with the potential to regulate these and other mammary-active genes, the Hedgehog (Hh) pathway (Task 3d in the revised SOW). Through study of a series of mutants, as well as expression studies, we have firmly established that this pathway is operative in the mammary gland and regulates mammary tissue structure. It has the potential of being considered a "master regulator" of mammary development.

The rational behind this Hh project has been to obtain a better and ultimately more clinically useful understanding of the genetic mechanisms underlying development of the normal breast and of the initiation, progression, and spread of breast cancer. The reasoning behind this question is as follows. The breast is a target organ for a variety of hormones. These, together with growth/differentiation factors, regulate the activities of the mammary cell. Unfortunately, this does not take us very far in understanding the biology of this interesting organ. Consider simply that other organs are also regulated by these same signaling molecules, but develop by quite a different pattern. The mammary gland itself varies enormously between species, between individuals, and of course in malignancy. How can this variation be accounted for, when the signals are the same? There must exist additional layers of genetic regulation that interpret these signals and give rise to particular patterns of development, or to neoplasia. How do we search for these developmental regulatory genes? In organisms such as *Drosophila*, where detailed genetic analysis is possible, mutations provide clues that have led geneticists to identify gene families that act as master regulators of cell fate. The discovery of these regulators has had an enormous impact on thinking in biology.

BODY OF NARRATIVE

HOXD-10: EXPRESSION AND FUNCTIONAL ANALYSIS (Task 4c in revised SOW).

Phenotype. In a recent report, Carpenter et al (1997) reported that targeted disruption of Hoxd-10 produces mice with hindlimb-specific defects in gait and adduction. To determine the underlying causes of this locomotor defect, mutant mice were examined for skeletal, muscular and neural abnormalities. Mutant mice exhibit alterations in the vertebral column and in the bones of the hindlimb. No major alterations in hindlimb musculature were observed, but defects in the nervous system were evident. There was a decrease in the number of spinal segments projecting nerve fibers through the sacral plexus to innervate the musculature of the hindlimb. Deletion of a hindlimb nerve was seen in some animals, and a shift was evident in the position of the lumbar lateral motor column. These observations suggest a role for the Hoxd-10 gene in establishing regional identity within the spinal cord.

Our initial phenotypic analysis of female mice homozygous for a disrupted *HoxD10* gene identified a defect in lactation. Pups from early litters of mutant females died from a lack of milk, but survived if pups were fostered with lactating wild type females. Lactational failure appears to be most pronounced in the first litter, and becomes less severe in subsequent litters, such that multiparous breeders are able to nurse successfully. At least

three hypotheses could explain this defect: 1) glands are developmentally delayed, 2) glands are defective in lobule-alveolar differentiation, and 3) glands are defective in functional differentiation (lactogenesis) such that milk production and secretion is compromised.

We have completed a program to characterize this phenotype and obtain insights into the functional activity of Hoxd-10. A breeding program was been set up to permit examination of mice at all stages of mammary development and during the lactation cycle. Using these techniques, we have shown that the defect lies in failure to complete functional differentiation resulting in compromised lactational efficiency. This defect is not fully penetrant in the Hoxd-10 null mice, but results in a high incidence of malnourishment, lack of normal weight gain, and a high incidence of infant mortality. In the second or third pregnancy lactational performance improves. In the 1997 progress report we provided a detailed summary of the expression of this homeotic gene in the mammary gland. This, taken together with the functional data mentioned above, completes this project and provides the first functional understanding of the role of this homeotic gene in the breast.

HEDGEHOG SIGNALING: REGULATION OF MAMMOGENIC GENES BY A HIGH-ORDER UPSTREAM REGULATORY PATHWAYS (Task 3d in the revised SOW).

Introduction. This objective, added in the 1997 revised SOW, is a direct outcome of two of our recent findings. First is the discovery of a mammary role for Hoxd-10, and second is cloning and expression of the IRX gene family in the gland that has been previously reported. In model systems, all of these genes are regulated, at least in part, by the hedgehog signaling pathway. An investigation of this pathway in the mammary gland was a logical and interesting extension of this previous research. Although a great deal of attention has recently been focused on hedgehog signaling in various developmental systems (reviews: Altaba, 1997; Hammerschmidt et al, 1997), it has not been studied in the mammary gland to our knowledge, and certainly nothing has been published.

An outline of hedgehog signaling in Drosophila, where it was first discovered and investigated in detail, is shown in Fig. 1. A single hedgehog ligand (*Hh*) is implicated in both short-range and long-range signaling through its receptor patched (*ptc*), whose activity is modified by another membrane protein, *Smo*. Hedgehog signaling is mediated by cubitus interruptus (*Ci*), a putative transcription factor that regulates downstream homeotic genes such as members of the Iroquois family (Gomez-Skarmetaet al, 1996), decapentaplegic, wingless, and patched itself. In the fly the hedgehog pathway has many essential functions, is active in many locations, and is reactivated at various times in development, from early segmentation and axial patterning to development of structures such as the wing, leg, eye, in the larva.

In vertebrates the pathway is not only conserved, but new family members have been added with a resulting increase in complexity and developmental plasticity (Fig 1). In mammals and birds hedgehog has been expanded to include Sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*), and Desert hedgehog (*Dhh*), whereas *Ci* has been expanded to include a family of three vertebrate transcription factors, the *Gli* genes, so named because of their initial identification and cloning from a glioblastoma (Rupperet et al, 1988). In addition to *Gli*, *Ptc* has recently been linked to both inherited and sporadic skin cancers, which include the basal cell carcinoma, the most common human cancer (Johnson et al, 1996). In spite of the expansion of family members, the signal transduction cascade appears to be remarkably conserved.

The multiple functions of the hedgehog pathway in vertebrate development are being studied in several laboratories and it is evident that, as in the fly, hedgehog regulation of developmental process occurs in many locations and in many developmental periods. One the most fully documented and elegant examples is in the developing limb, where Shh secretion regulates patterning of the anterior-posterior axis (Marigo et al, 1996). Numerous

developmental genes have been shown to be regulated by this pathway such as IRX, Hox, TGF-Beta, BMP, FGF, and even the recently discovered parathyroid-related-protein (PTRP), which in gene-targeting experiments has recently been shown to be essential for embryonic growth of the mammary gland. The above list reads like a litany of genes and cell products that are known to be important to mammary development and cancer. Thus our discovery that the hedgehog pathway is active in the mammary gland has far-reaching implications.

Research Plan. Our approach to studying the hedgehog pathway in the mammary gland involves three strategies. The first is expression, using appropriate combinations of molecular probes and antibodies as available. Second, we have obtained mutants of various components of the pathway and are well into a breeding program that will enable us to examine phenotypic changes in development, function, or neoplastic potential of the gland. These studies will make use of our nude colony, enabling us to examine mutant and control tissues in a uniform physiological environment.

Patterns of expression of Hh signalling components. In the 1997 Progress Report several of spatial and temporal expression patterns of Hh signaling components were described and illustrated in considerable detail. In 1998 this work was repeated and in some cases extended, but in no instance were contradictory results obtained. In order to summarize this very large amount of data we have prepared many of our results in graphic form (Fig. 2). In general most expression was robust, highly specific, and in all cases developmentally regulated. When considered in relation to the functional data summarized in the following sections, the expression is quite consistent with functional activity and again provides compelling evidence for the activity of this regulatory pathway.

PATCHED-1*:* This mutation in the primary receptor for the Hedgehog pathway creates defects in mouse mammary gland development caused by conditional haploinsufficiency (at press, *Development*)

Summary. We report investigation of the role of the *Patched-1* (*Ptc1*) hedgehog receptor gene in mammary development and neoplasia. Haploinsufficiency at the *Ptc1* locus results in severe histological, but only minor morphological, defects in mammary glands of heterozygous postpubescent virgin animals. Defects are mainly ductal hyperplasias and dysplasias characterized by cellular impaction of ductal lumens. Haploinsufficiency is conditional in that lesions are reverted during late pregnancy and lactation but return upon involution and gland remodeling. Unlike most mouse mammary hyperplasias and tumors, *Ptc1*-induced lesions are not stable upon transplantation into an epithelium-free fat pad. This transplant behavior is similar to that of human basal cell carcinoma (BCC), which can be *Ptc1*-induced, when transplanted into athymic mice. Mammary expression of *Ptc1* mRNA is primarily epithelial and developmentally regulated. Data demonstrate a critical mammary role for at least one component of the hedgehog signaling network and suggest that *Ptc1* may act as a mammary tumor suppressor gene.

Introduction. A compelling reason to study the hedgehog signaling network in the mammary gland, in addition to its developmental interest, is the issue of breast cancer. With respect to a possible role in mammary tumorigenesis, several of the genes in the mammalian hedgehog signaling network have been identified as either protooncogenes or tumor supressor genes. A number of these genes, including *Ptc1*, *Smo*, *Shh* and *Gli1*, contribute to the development of skin cancers, most notably basal cell carcinomas (BCC) (Dahmane et al., 1997; Fan et al., 1997; Ingham, 1998a; Oro et al., 1997; Reifenberger et al., 1998; Xie et al., 1998). In addition to skin lesions, *Ptc1* has also been causally implicated in the development of medulloblastomas (brain tumors) and other soft tissue tumors (Goodrich et al., 1997; Hahn et al., 1998). While *Ptc1* mutations have been

identified in a small fraction of human breast cancers (Xie et al., 1997), no general role for the gene has been established in the mammary gland.

Of the two known hedgehog receptors, *Ptc1* is most fully characterized. Animals homozygous for targeted disruption of *Ptc1* show early embryonic lethality (around embryonic day 9.5) with, among other alterations, severe defects in nervous system development accompanied by changes in neural cell fates. Heterozygous animals can also show defects including skeletal abnormalities, failure of neural tube closure, medulloblastomas (brain tumors), rhabdomyosarcomas, and strain-dependent embryonic lethality (Goodrich et al., 1996; Hahn et al., 1998). The severity of these defects, even in heterozygotes, and the central role *Ptc1* plays in hedgehog network function made *Ptc1* a good candidate gene upon which to focus attention. We have investigates the role of the *Patched-1* (*Ptc1*) hedgehog receptor gene in mammary gland development and neoplasia. *Ptc1* expression is both developmentally regulated and cell type specific. Wild type levels of *Ptc1* function are essential for proper mammary histogenesis, with heterozygous animals developing ductal hyperplasias resembling human ductal carcinoma in situ (DCIS). These lesions are reversible during pregnancy and lactation, allowing normal secretory function. Our finding that a central component of the hedgehog signaling network is active in the mammary gland and that its function is conditionally required for mammary histogenesis offers the possibility that this regulatory network will provide a genetic framework upon which to integrate many of the previously identified mammary developmental control genes and signaling pathways. In addition, altered hedgehog network function could provide novel insights into mammary cancer initiation and progression.

Materials and Methods. Two breeding pairs of mice heterozygous for a disrupted *Ptc1* gene were used to initiate a breeding colony and have been previously described (Goodrich et al., 1997). The original *Ptc1* mutation was maintained in a 129Sv:C57/Bl6 background with subsequent backcross to B6D2F1. In our laboratory, the mutation was likewise maintained in a B6D2F1 background by serial backcross but is still in a mixed background (as evidenced by segregation of coat color markers) which precluded transplants between animals (see below). Genotyping was performed by PCR as per Goodrich (1997). In all other respects, the materials and methods are as previously described in earlier reports.

Targeted disruption of the *Ptc1* gene results in defective tissue organization during virgin development. In situ hybridization demonstrated that *Ptc1* expression was both spatially and temporally regulated during mammary development, suggesting a functional role. To determine whether or not disruption of the *Ptc1* gene resulted in developmental defects in the mammary gland, glands were examined from several stages of development. No alterations were observed in overall patterning of the mammary tree at 3 weeks of age (data not shown). At 5 weeks of age, terminal end buds in wild type animals appeared normal in whole mount preparations of whereas up to approximately 30% of terminal end buds in heterozygous animals appeared misshapen or disrupted. Disruption of TEB at 5 weeks did not lead to alterations in ductal patterning in adult animals at 10 weeks of age. No morphological distinctions in whole mount preparations could be made between wild type and heterozygous glands, leading us to think initially that the Ptc heterozygote had no mammary phenotype.

Upon histological analysis, however, we noted severe ductal dysplasias and hyperplasias in 100% of heterozygous animals by 5 weeks of age. While not apparent in glands taken from 3 week old wild type and heterozygous animals (Figure 3A and 3B, respectively), severe histological abnormalities were observed at 5 weeks when compared with wild type controls (Figure 3C versus 3D). Normally, multilayered lumenal epithelial cells (body cells) of the TEB thin to a monolayer as the subtending duct is established (Figure 3C). However, in some ducts of 5 week heterozygous animals, the lumenal epithelium remained multilayered and, in some cases, the lumenal space was completely occluded by epithelial cells (Figure 3D). Condensation of the periductal stroma around the

neck of the TEB appeared altered in some cases such that adipocytes were included within the condensate and condensation appeared to occur at an unusual distance away from the duct (Figure 3D). At higher magnification, body cells of wild type end buds appear well ordered and cap cells form a distinct, organized layer as they differentiate into myoepithelial cells (Figure 3E). By contrast in some endbuds of heterozygous animals, body cells were disordered (Figure 3F) and the cap cell layer was visibly altered (Figure 3F).

Ductal impaction observed at 5 weeks of age is even more pronounced in glands from 10 week old animals. Whereas wild type ducts show a clear lumen within a monolayer of lumenal epithelial cells (Figure 3G), a majority of ducts in glands from heterozygous are partially or completely impacted with cells (Figure 3H) as detected by examination of serial sections through entire ducts. Interestingly, some ducts appear relatively unaffected. Cells in impacted ducts are not monomorphic with respect to nuclear morphology and can include large cells with round nuclei and clear cytoplasm suggesting that multiple epithelial subtypes contribute to the lesions.

To begin to address which cell types contribute to ductal lesions and to further characterize cells within the lesions, staining with propidium iodide (nuclear stain) and phalloidin (actin stain) was performed to assay for alterations in actin localization in the myoepithelial and epithelial cell layers. In wild type ducts (Figure 3I), actin staining clearly identifies the myoepithelial cell layer as well as the terminal web and microvilli at the apical (lumenal) surface of lumenal epithelial cells. Faint actin staining is also observed on the lateral surfaces of lumenal cells. In affected ducts of heterozygous animals (Figure 3J), myoepithelial cells do not appear to contribute to the cell population of the lesions but remain associated with the basal lamina surrounding the impacted ducts. By contrast, actin staining within the lesion is generally disorganized but can be observed at the apical cell surface around microlumens formed by circular clusters of epithelial cells (Figure 3J). Data suggest that only lumenal epithelial cells contribute to the lesions and that cells can become polarized, albeit inappropriately, around microlumenal spaces within the lesions.

***Ptc1*-induced lesions are reversible during pregancy and lactation**. Given the severity of the mammary phenotype in virgin *Ptc1* heterozygotes, the question arises: why does cellular impaction of ducts in mature animals not impair their ability to lactate? To investigate, we examined glands at various stages of pregnancy, lactation and involution. By histological analysis, many ducts in early pregnancy remain filled, or nearly filled, with cells and are qualitatively similar to those of mature virgins (data not shown). However, by late pregnancy and lactation most ducts of heterozygotes show phenotypic reversion toward a wild type histoarchitecture, becoming cleared of epithelial blockages with duct walls thinned to form a single layer of lumenal epithelial cells, with only sporadic cellular impaction of ducts remaining evident. Ducts in heterozygous animals remain open in early stages of involution (data not shown) and in late involution as do wild type ducts but elements of the impacted phenotype are re-established in some ducts by late involution (14 days) with an occasional observance of severe stromal overgrowth.

These results indicate that the hedgehog network is strongly influenced by physiological changes that occur during pregnancy and maintained during lactation and suggest that the network may interact with hormone- or growth factor-mediated signal transduction networks. Since levels of several mammatropic hormones and growth factors (e.g. estrogen, progesterone, prolactin, TGF-ß family members) are dramatically altered during these stages, and disruption of each of these signaling networks independently disrupts gland development and function, identification of the network(s) involved in phenotypic reversion is likely to be complex.

***Ptc1*-induced lesions are not stable upon transplantation into cleared fat pads of wild type recipients**. With respect to a contributory role in breast cancer, an important question concerning the nature of the *Ptc-1*-induced lesions is whether or not the lesions represent a preneoplastic or neoplastic state. To determine whether *Ptc1*-induced

lesions are stable or undergo neoplastic progression upon transplantation, wild type and heterozygous mammary epithelium were contralaterally transplanted into epithelium-free (cleared) fat pads of athymic mice and allowed to regenerate a ductal tree for 6 weeks to 8 months.

Donor epithelium from wild type animals was normal whereas heterozygous donor epithelium from the region surrounding the transplanted area showed mild-to-severe histological defects. Upon transplantation, wild type epithelium produced normal ductal outgrowths, as expected. Epithelium transplanted from affected heterozygous animals were also histologically normal even after 8 months posttransplantation. These data indicate that *Ptc1*-induced lesions are not stable upon transplantation under these conditions and suggest that *Ptc-1* lesions may represent an early or contributory step in neoplastic progression. Results further suggest that *Ptc1* function may be required in the stroma (or in both epithelium and stroma) for transplanted heterozygous epithelium to recapitulate the mutant phenotype observed in virgin animals but do not rule out the possibility of hedgehog network interactions with systemic factors.

Ptc1 is differentially expressed in mammary epithelial cell types. To further investigate developmental regulation suggested by Northern analysis and to determine which cell types express *Ptc1*, exhaustive *in situ* hybridization was performed against mammary tissue at various developmental stages. These data are summarized in Fig. 2.

Conclusions. By expression and functional analysis, we have shown that in gene-targeted mutants the *Ptc1* hedgehog receptor is not only developmentally regulated at the mRNA level, but is also conditionally required for proper histogenesis during virgin development and late-stage involution. *Ptc1*-induced lesions appear to be due primarily to failure of body cells of the terminal end bud to thin to a single cell layer in the subtending duct. This failure is compounded by progressive duct wall thickening with cellular impaction of the lumen in a majority of mammary ducts by 10 weeks of age. Cellular impaction is reversible during late pregnancy and lactation allowing successful milk secretion to occur. Mammary lesions are not stable on transplantation suggesting that *Ptc1* function may also be required in the stroma. To our inowledge this is the first mutation in mice associated with tissue construction and maintenence.

An unusual aspect of the *Ptc1* phenotype is that it illuminates a distinction between the genetic regulation of two fundamental aspects of mammary ductal development, namely pattern formation and ductal morphogenesis. The patterning of the branched, mammary ductal system and the development of its component ducts have tacitly been considered interdependent; without proper ductal morphogenesis, overall gland architecture would be altered. The *Ptc1* phenotype demonstrates genetic separation of these two developmental processes. Ductal patterning is a highly regulative process that results from end bud bifurcations and turning maneuvers in response to local environmental signals from the stroma and from nearby mammary epithelium. In the *Ptc1* animals, a normal branching pattern is established even though the internal structure of individual ducts is severely disrupted indicating that reception and interpretation of these environmental signals is not impaired.

GLI-2*:* Disruption leads to severe mammary defects in both the null and heterozygote

Introduction *Gli-2* is one of three transcription factors in the vertebrates that mediate hedgehog signaling and regulate downstream targets. Very little is known about the specific roles of the mammalian *Gli* transcription factor genes (*Drosophila Ci* homologs) in hedgehog signaling. One recent model postulated that the GLI1 protein took on the transcriptional activation functions ascribed to*Drosophila* Ci_{act} and that GLI3 protein took on the transcriptional repression function ascribed to Ci_{rep} with GLI2 modulating the function of GLI1 and GLI3 in an unspecified manner [Dahmane, 1997 #1987]. Subsequent

mutational analysis of each of the three genes was not consistent with this model in that disruption of *Gli-1* shows no detectable defects, even in homozygotes, whereas disruptions of *Gli-2* and *Gli-3* are both perinatal lethal and lead to overlapping, but distinct, developmental defects (Table 1)[Matise, 1998 #49][Mo, 1997 #143]. These data suggest that *Gli-2* and *Gli-3* can have overlapping and redundant function depending on the structure being examined. By contrast, a competing model suggests the activity of GLI proteins may be regulated by proteolytic processing similar to that observed for their *Drosophila* counterpart CI [Biesecker, 1997 #1514]. Under this model, GLI proteins may act as both transcriptional activators or repressors depending on their cleavage state.This model is supported by recent data that different two forms of GLI2 act as a transcriptional activator and a transcriptional repressor, respectively [Tanimura, 1998 #1483]. Together, these data suggest that *Gli-2* and *Gli-3* are the primary transcriptional regulatory genes that mediate expression of target genes in mammals and that, in some tissues, these two genes can act coordinately, either in the same or opposite directions, to regulate development.

Materials and Methods. Mice heterozygous for targeted inactivation of *Gli-2*. generously provided by C.C.Hui. Unlike homozygous *Ptc-1* mutants, which display very early embryonic lethality, *Gli-2* -/- mutants die shortly before birth. Although the mammary glands of these null mice cannot be examined during postnatal development, we successfully "rescued" the mammary glands by whole-gland transplants by removing the embryonic gland and transplanting it between the skin and body wall of a host animal (in this case, immunocompromised). The glands were allowed to develop in this wild type hormonal background for several weeks. Although these transplants do not attain their full normal size, they do undergo full organotypic development.

Homozygous disruption leads to severe mammary defects. Glands from wild type and *Gli-2* homozygous late-stage embryos were examined using this technique. Wild type glands grew normally with unperturbed histoarchitecture. By contrast, glands from *Gli-2* homozygous donors showed multiple defects including hyperplastic or distended ducts and severely altered histoarchitecture that closely resembles human micropapillary ductal carcinoma in situ (Fig 4A). These transplant experiments indicate that the mammary defects observed were intrinsic to the mammary gland and were not significantly effected by environmental or systemic influences. In this respect, the *gli-2* phenotype is simpler than that of *Ptc-1*, in which the phenotype appears to reflect the interaction between the local environment of the nude mouse and the mutated gene in the mammary gland. Another conspicuous difference is that the *gli-2* defects are even more pronounced and bizarre, perhaps reflecting the homozygous null genotype.

Heterozygous disruption of Gli-2 leads to focal mammary hyperplasia. *Gli-2* heterozygotes have been examined at several developmental stages. Mammary hyperplasias and dysplasias are detectable as early as 5 weeks postpartum and progress to form easily identifiable focal lesions by 10 weeks postpartum. Histologically, advanced lesions are highly disorganized with loosely adherent epithelial cells impacting the ductal lumen similar to the phenotype in *Ptc-1* heterozygotes. Interestingly, ducts removed from the lesions can also show dramatic alteration of histoarchitecture while appearing normal in whole mount preparations. Here again, as in the *Ptc-1* mutant, there is a disconnection between branching morphogenesis at the organ level, which is normal in appearance, and histoarchitecture, which is severely disrupted. These lesions are illustrated in Fig. 4B.

CONCLUSIONS. Although there are distinct differences between the *ptc-1* and the *gli-2* phenotypes, there is a gratifying degree of overlap -- to be expected in mutations in different components of the same signaling network. The more focal lesions in *gli-2* must now be examined for their transplantability and tumorigenic potential.

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Sarajane Ross, Graduate Student
Charles Sugnet, Staff Research Associate
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FIGURE LEGENDS

Fig. 1. (A) Hedgehog signaling in fruitfly (simplified). Known functions are depicted for several hedgehog signaling network proteins. Activating functions are noted with arrowheads; inhibitory interactions are noted with lines. Genes under transcriptional control of CI are noted in italics. **(B)** General model for the hedgehog signaling network in the vertebrates (simplified). Known functions are depicted for several hedgehog signaling network proteins. Activating functions are noted with arrowheads; inhibitory interactions are noted with lines. All network genes shown are expressed in the mammary gland.

Fig. 2. Summary of in situ hybridization expression data for Ptc-1, Ihh,and Gli-2 and correlation with phenotypic alterations in mutant strainsthrough mammary development. Predominant morphological features present inmammary glands at different develop amental stages are denoted by coloredhorizontal bars (upper section). Phenotypic alterations in Ptc-1 and Gli-2knockout strains are noted by color for the altered mammary structure(middle section). Subjective evaluation of relative expression levels in predominant mammary tissue compartments and cell types are also shown by color (lower section).

Fig. 3. Histological comparison of glands during virgin development. Animal developmental stage is shown along the left edge of the figure; genotype of the animal from which the gland is derived is shown at the top of each column of panels. Panels A-H are stained with hematoxylin and eosin; panels I-J are stained with phalloidin (yellow-green, actin) and propidium iodide (red, nuclei). Ductal lumens are denoted by red asterisks (*); Adipose stroma is denoted by a red letter "s" A) Longitudinal section through a mammary duct. Lumenal epithelium is generally a monolayer of darkly staining cells surrounding the ductal lumen. Eosinophilic (pink) periductal stroma adjoins the duct and consists mainly of fibroblasts. Bar = 80 μm B) Mammary duct which is indistinguishable from its normal counterpart. Bar = 80 μm. C) Terminal end bud with characteristic body cell layer composed of 3-6 layers of epithelial cells thinning to a monolayer surrounding an well-defined lumen in the subtending duct (red arrow). A thin, uniform layer of condensing periductal stroma is shown at the neck of the TEB and along the duct . Bar = 200 μm. D) Terminal end bud. Body cell layer fails to thin to a monolayer in the subtending duct (red arrow) resulting in ductal occlusion. Stromal condensation may occur at unusual distances from the TEB and can also appear disrupted with the inclusion of adipocytes within the condensate (black asterisks). Bar = 200 μm. E) Terminal end bud at increased magnification. Body cell layer appears well ordered surrounded by a well-defined monolayer of cap cells (black arrow). Bar = 80 μm. F) Terminal end bud at increased magnification. Body cell layer appears less well organized with a clearly disrupted cap cell layer (black arrows). Note the unusual inclusion of adipocytes (black asterisks) within the condensed stroma at the tip of this end bud. Bar = 80 μm G) Normal mammary duct. Bar = 80 μm. H) Severely affected mammary duct showing complete occlusion by epithelial cells. Bar = 80 μm. I) Normal mammary duct. Lumen is denoted by a white asterisk. A uniform layer of myoepithelial cells is identifiable (white arrows) as a line of yellow cells lining the outer surface of the duct. Bar = 80 μm. J) Severely affected mammary duct showing complete occlusion by epithelial cells. The myoepithelial cell layer (white arrow) appears unaffected. Clusters of epithelial cells which form microlumens within the ducts can be identified (white circles) with inappropriate actin localization at the microlumenal surface. Bar = 80 μm.

Figure 4. **(A)** The *Gli-2* null phenotype. A) Representative transplant gland from a *Gli-2* homozyous donor. Ducts show altered branching and unusual termini. B) Representative transplant gland from a wild type donor showing normal branching and duct termini. C. Histology of ducts from *Gli-2* null gland showing papillary epithelial structures. D) Histology of human micropapillary ductal carcinoma in situ. A and B hematoxylin only; C and D hematoxylin and eosin. **(B)**. Histological analysis of representative mammary lesions from *Gli2* heterozygotes and comparison with a similar human lesion. Hematoxylin and eosin staining (except A, hematoxylin only). Ductal lumens are noted by a blue letter L; Adipose stroma is noted by a blue letter S. A) Whole gland preparation showning a representative hyperplasia (red arrow) from a multiparous female and adjacent normal appearing ductal and alveolar structures (white arrow). B) Histological section of lesion in A. Note the epithelial and stromal proliferation and disorganization (arrow) and eosinophilic inclusions (asterisk) (probably keratin). C) Dysplastic duct adjacent to the cell mass in A. Duct walls appear multilayered with loosely associated cells in the lumen

(arrow). D) Lobule-alveolar hyperplasia budding off duct with multiple layers of epithelial cells (arrow). E) Normal duct showing a single layer of epithelial cells lining the duct (arrow). Compare with B,C and D. F) Human ductal carcinoma in situ [Fechner, 1990 #2153]. Compare with C.

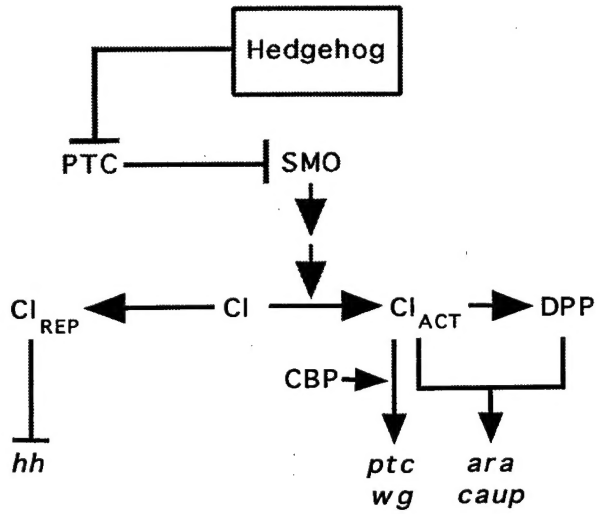

Hedgehog
PTC
SMO
Cl REP
Cl
Cl ACT
DPP
CBP
hh
ptc
wg
ara
caup

Fig 1A

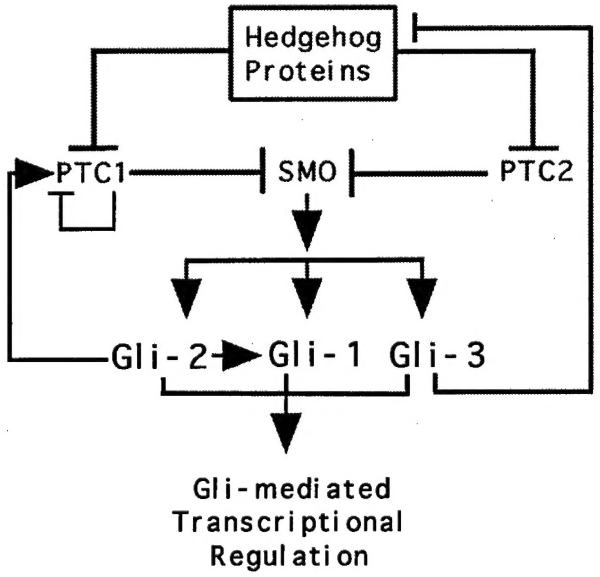
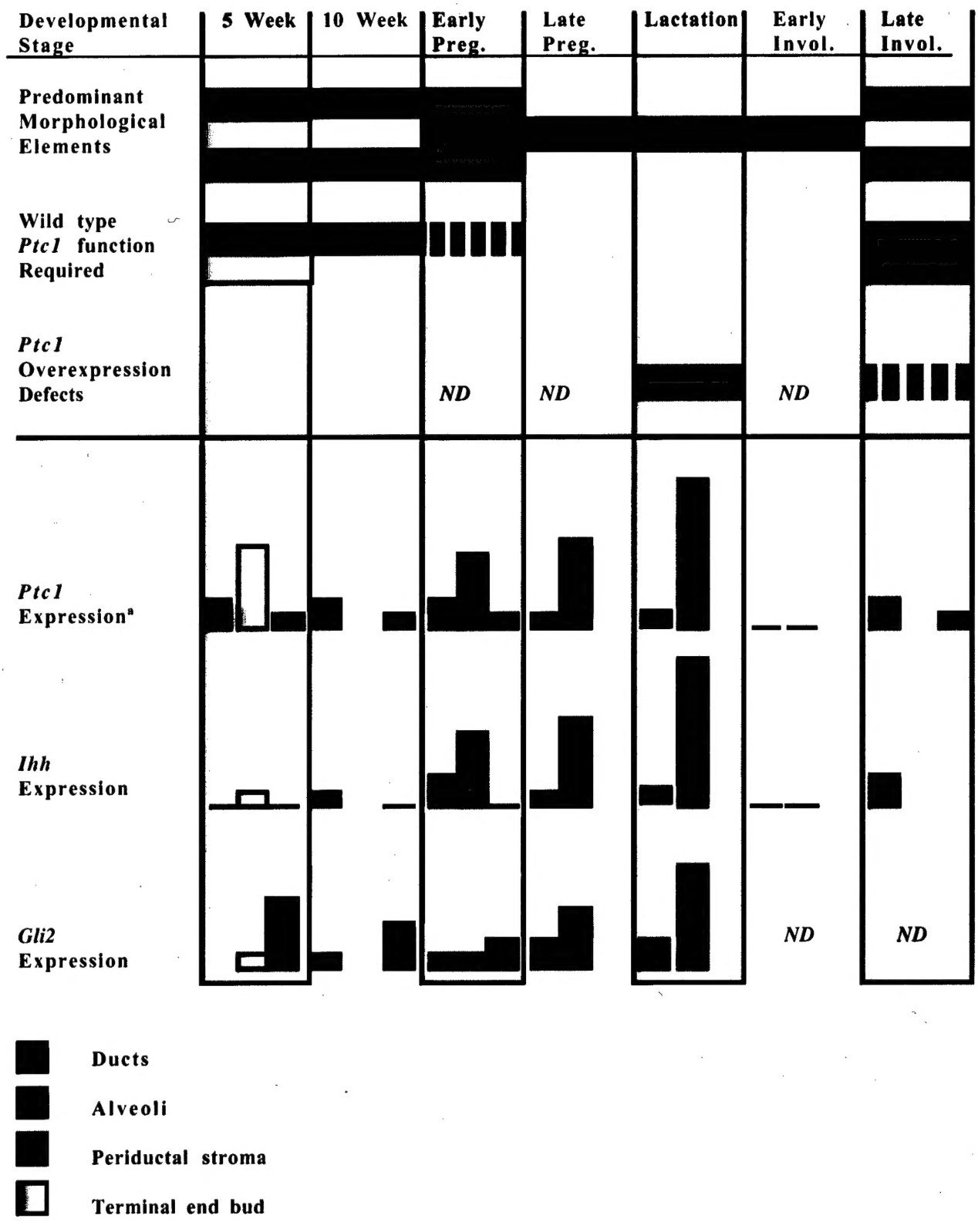
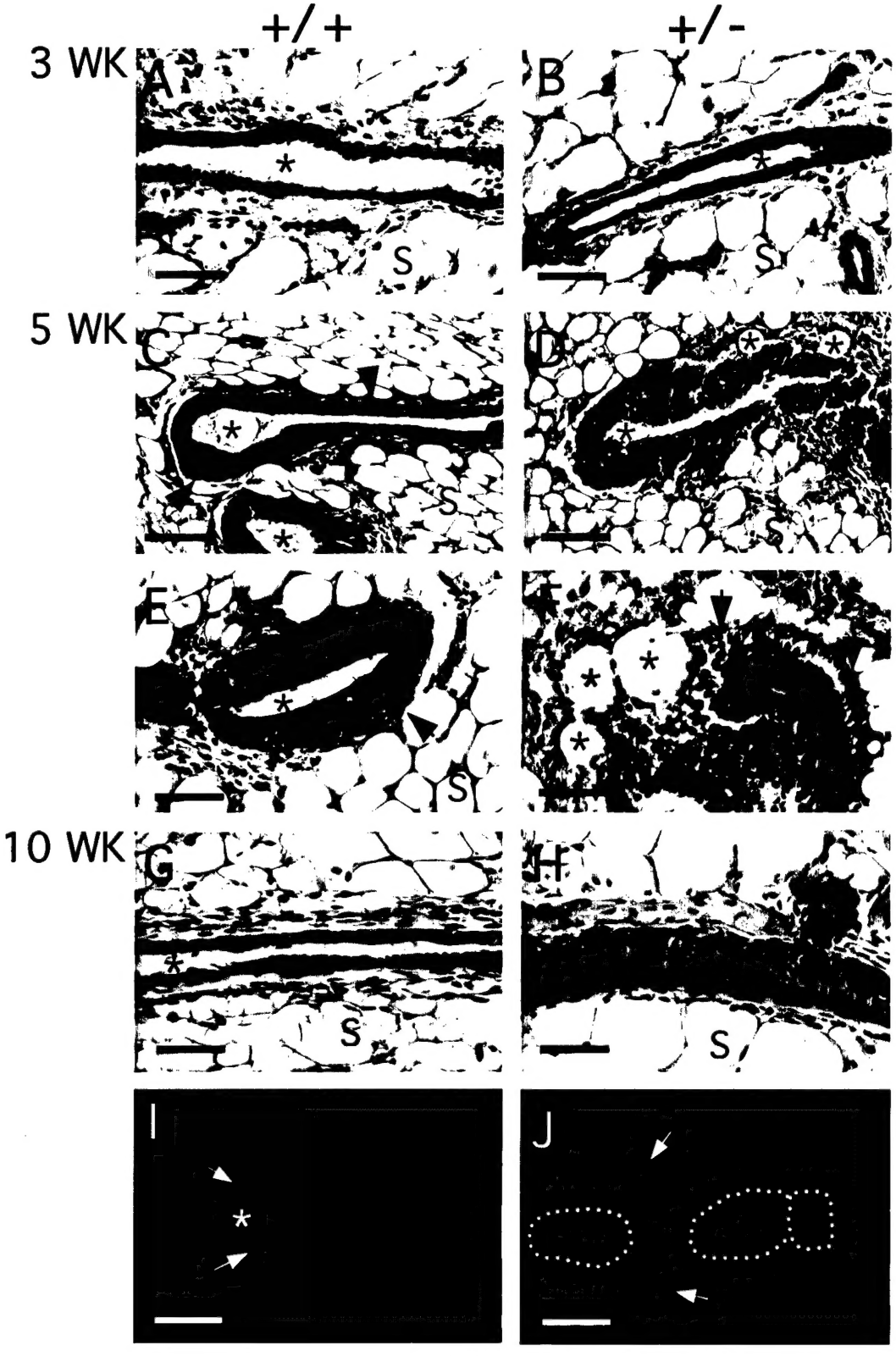

Hedgehog
Proteins
PTC1
SMO
PTC2
Gli-2
Gli-1
Gli-3
Gli-mediated
Transcriptional
Regulation

Fig. 1B


Developmental Stage
5 Week
10 Week
Early Preg.
Late Preg.
Lactation
Early Involution
Late Involution
Predominant Morphological Elements
Wild type Ptc1 function Required
Ptc1 Overexpression Defects
ND
ND
ND
Ptc1 Expression[a]
Ihh Expression
Gli2 Expression
ND
ND
Ducts
Alveoli
Periductal stroma
Terminal end bud


+/+
+/-
3 WK
5 WK
10 WK
A
B
C
D
E
F
G
H
I
J
S

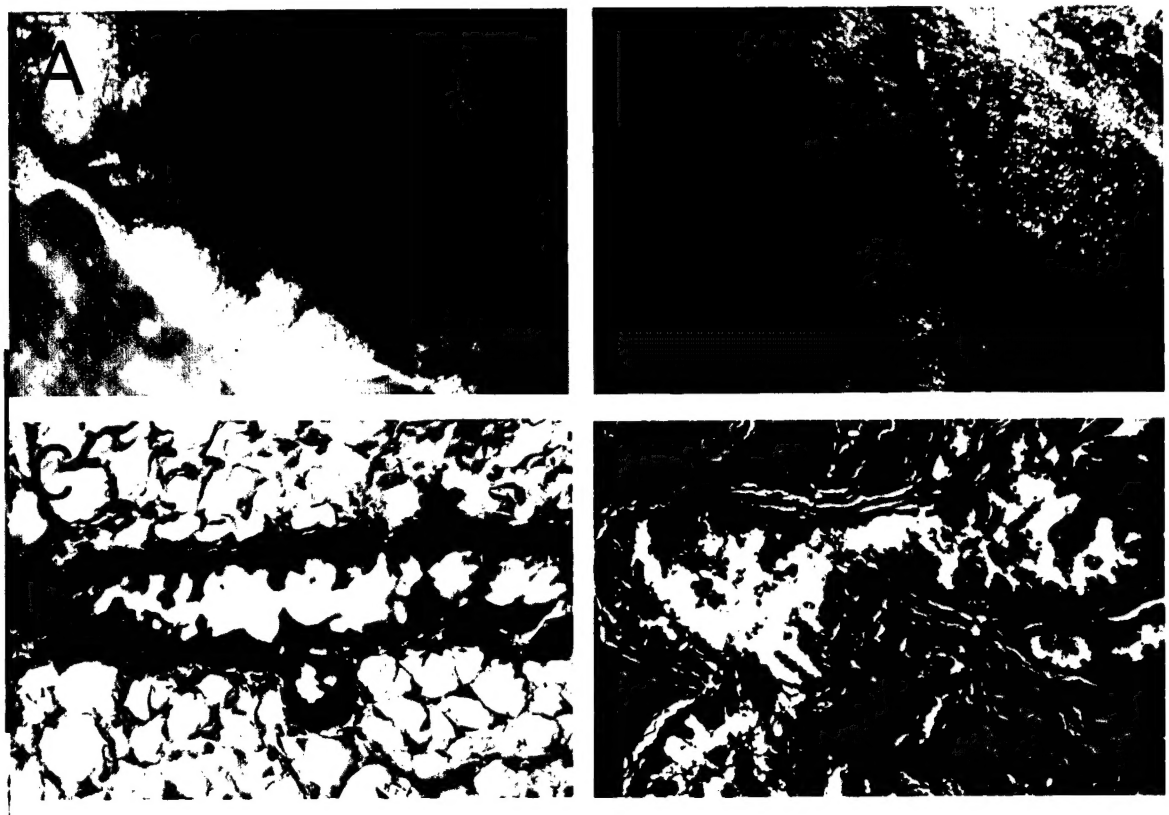


Fig 4A

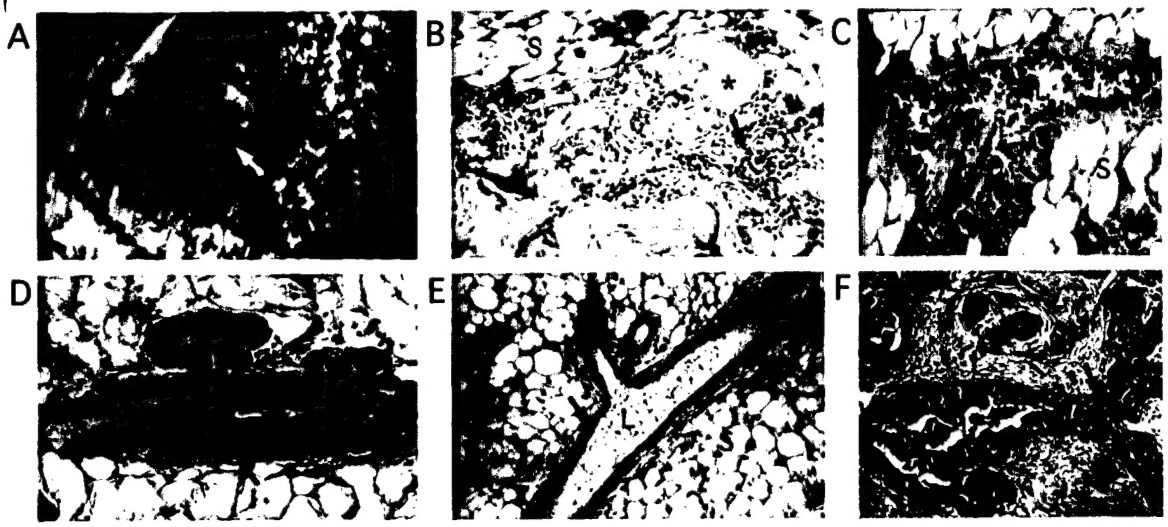


Fig 4B